Supplemental Information:

Nrf1 can be processed and activated in a proteasome-independent manner

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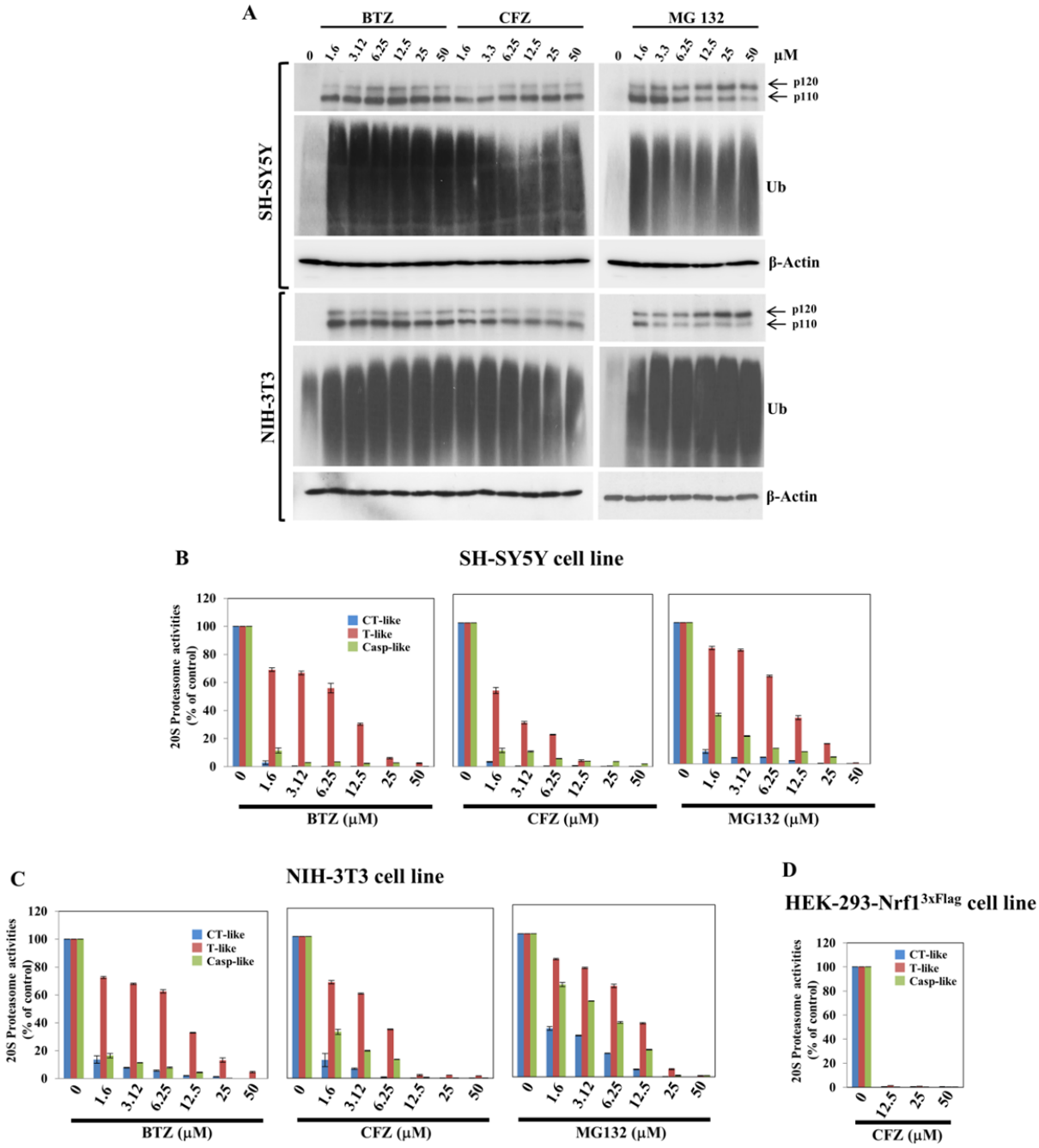

Figure S1. Effect of proteasome inhibitors on different cell lines.
(A) SH-SY5Y human neuroblastoma and NIH-3T3 mouse fibroblast cells were treated for 16 hr with different concentrations of bortezomib (BTZ), carfilzomib (CFZ) or MG132 as indicated. The whole cell lysates were immunoblotted to detect p120 and p110 forms of Nrf1. β-actin served as a loading control. SH-SY5Y cells in (B) and NIH-3T3 cells in (C) were treated for 4 h with different concentrations of BTZ, CFZ or MG132 as indicated. HEK-293-Nrf1^{3xFlag} cells in (D) were treated for 1 h with CFZ. Chymotrypsin-like (CT-like), tryspin-like (T-like) and caspase-like (Casp-like) activities of the 20S proteasome were measured and these values were normalized to cell viability and expressed as percentage of activities relative to control cells. Error bars denote SD (n = 3).

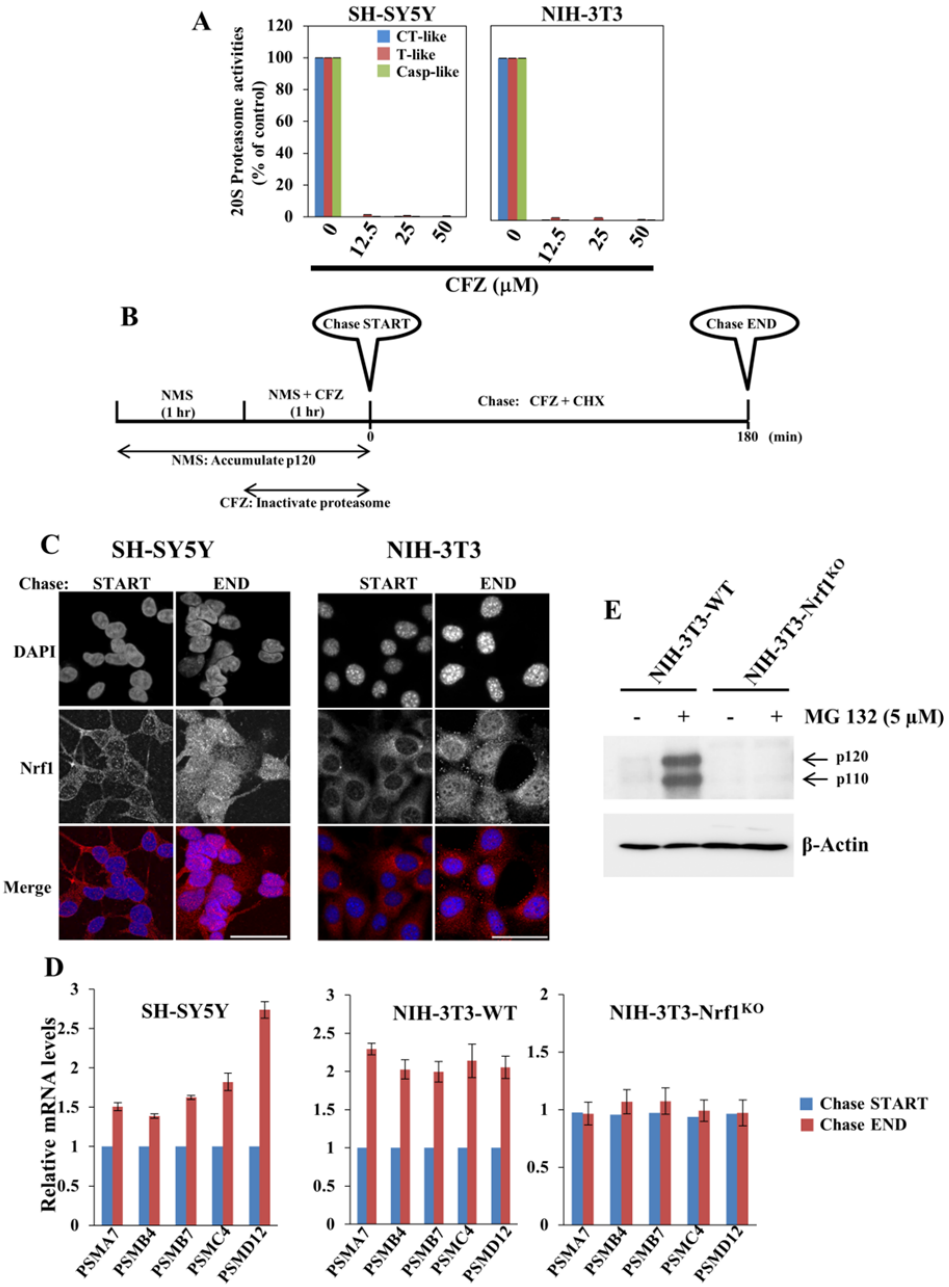

Figure S2. Processed Nrf1 can migrate to the nucleus and activate PSM gene transcription when proteasome is completely inactivated.

(A) SH-SY5Y and NIH-3T3 cells were treated with CFZ for 1 h and chymotrypsin-like (CT-like), tryspin-like (T-like) and caspase-like (Casp-like) activities of the 20S proteasome were measured and these values were normalized to cell viability and expressed as a percentage of activities relative to control cells. (B) Schematic representation of the pulse-chase experiment is shown. SH-SY5Y or NIH-3T3 cells were pretreated for 2 h with 10 μM NMS. During the second hour, the cells were additionally exposed to 12.5 μM CFZ (which completely inhibits all three active sites of the proteasome). After washing out the NMS, the cells were then chased with 12.5 μM CFZ and 100 μg/mL CHX. The cells were harvested at the sample collection points indicated as 'Chase START' and 'Chase END'. These samples were used to track Nrf1 by immunofluorescence in (C) and assess Nrf1-mediated *PSM* gene transcription by quantitative RT-PCR in (D), where NIH-3T3 cells with Nrf1 knocked out (NIH-3T3-Nrf1^{KO}) were also included as a control. Error bars denote SD (n = 3). (E) Wild-type (NIH-3T3-WT) and Nrf1 knockout (NIH-3T3-Nrf1^{KO}) cell lines were treated with 5 μM MG132 for 6 h. The whole cell

lysates were immunoblotted to detect p120 and p110 forms of Nrf1. β-actin served as a loading control.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture and RNA interference

HEK-293-Nrf1^{3xFlag} (described in [S1]), SH-SY5Y, and NIH-3T3 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), penicillin and streptomycin (Invitrogen). HeLa cells were cultured in Basal Iscove media containing 10% fetal calf serum, 2 mM L-glutamine (Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Pan-Biotech, Aidenbach, Germany). All of the above cell lines were grown at 37°C and 5% CO_2 in a humidified atmosphere. For RNA interference experiments, SMARTpool siRNAs were purchased from Dharmacon and transfected using siRNA X-treme gene reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions.

CRISPR-Cas9-mediated deletion of Nrf1

The CRISPR construct targeting mouse Nrf1 was based on a 20-mer sequence (TTCCGCACCCTCGCCTGACC) present in the coding region of the gene and was cloned in to the lentiCRISPR v2 vector (gift from Feng Zhang; Addgene plasmid # 52961 [S2]). The lentiviral supernatant produced with the CRISPR construct was used to infect NIH-3T3 cells. These cells were selected in puromycin to generate the Nrf1 knockout cell line (NIH-3T3-Nrf1^{KO}).

Immunoblot analysis

Cells treated with bortezomib, carfilzomib (L.C. Laboratories), or MG132 (Selleck Chemicals) were directly lysed in 1x Laemmli sample buffer followed by brief sonication to disrupt the

viscous genomic DNA. For Figure 1D, cells were processed for immunoblotting as described previously [S3]. Detection was performed using antibodies specific for TCF11/Nrf1 (8052S, Cell Signaling), β-actin (A5441, Sigma-Aldrich), β7/PSMB4 (BML-PW8890-0025, Enzo), RPN10/PSMD4 (BML-PW9250-0025, Enzo), β-tubulin (MMS-410P, Covance), and Ubiquitin (Z0458, Dako or ADI-SPA-200, Enzo).

Pulse-chase assay

HEK-293-Nrf1^{3xFlag} cells were starved for an hour in Methionine-free DMEM (Invitrogen) to deplete intracellular Methionine reserves. The cells were then pre-treated for 2 h with 10 μM NMS-873 (Sigma-Aldrich) to accumulate Nrf1 p120. During the second hour, the cells were additionally exposed to 12.5 μM carfilzomib to completely inactivate the proteasome and pulse-labeled with 50 μM L-azidohomoalanine (Click Chemistry Tools). NMS-873 was then washed out and the cells were subjected to a chase in the presence of 12.5 μM carfilzomib, 100 μg/mL cycloheximide (VWR) and excess of L-methionine (Sigma-Aldrich). The cells were then harvested at different time points and the lysates were subjected to immunoprecipitation with anti-Flag beads (Sigma-Aldrich). The resulting immunoprecipitants were labeled with Biotin-PEG4-alkyne (Click Chemistry Tools). Biotin-labeled species were detected by immunoblotting with Neutravidin-HRP (Pierce Biotechnology).

Immunofluorescence microscopy

Cells grown on coverslips were fixed and permeabilized in -20ºC methanol containing 0.1% triton X-100 for 10 min. Cells were washed 3 times with PBS and blocked with 2% BSA for 2 h followed by incubation with anti-Nrf1 antibody (8052S; Cell Signaling) for 2 h in 2% BSA at room temperature. The cells were then exposed to Alexa fluor 594 conjugated secondary antibody (A11037; Life technologies) for 1 h in 2% BSA and washed 3 times with PBS. Nuclei were stained with 1 mg/ml DAPI (D1306; Life Technologies) in blocking buffer for 30 min.

Cells were then mounted using Vecta shield mounting media (Vector laboratories) and images were obtained using a Zeiss LSM700 confocal microscope with 63X oil immersion objective.

Quantitative reverse transcription PCR

RNA was isolated using the RNAeasy kit (Qiagen). cDNA was prepared using the iScript kit (Bio-Rad) according to the manufacturer's recommendations. This cDNA was then subjected to Quantitative PCR using iTaq universal SYBR Green supermix (Bio-Rad). The primers used to quantify proteasome subunits (PSM) and GAPDH mRNA levels have been described previously [S4].

Cellular proteasome activity assay

Cells were subjected to freeze-thaw lysis in TE buffer (20mM Tris pH 8 and 5 mM EDTA). Proteasome chymotrypsin-like, trypsin-like and caspase-like activities in the lysates were measured using the fluorogenic peptide substrates Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC respectively in the presence of 0.035% SDS as described previously [S5]. These values were normalized to the number of viable cells as determined by the cell-titer glo luminescence assay (Promega).

SUPPLEMENTAL REFERENCES

S1. Radhakrishnan, S.K., den Besten, W., and Deshaies, R.J. (2014). p97-dependent retrotranslocation and proteolytic processing govern formation of active Nrf1 upon proteasome inhibition. eLife *3*, e01856.

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S3. Steffen, J., Seeger, M., Koch, A., and Kruger, E. (2010). Proteasomal degradation is transcriptionally controlled by TCF11 via an ERAD-dependent feedback loop. Mol. Cell *40*, 147-158.

S4. Radhakrishnan, S.K., Lee, C.S., Young, P., Beskow, A., Chan, J.Y., and Deshaies, R.J. (2010). Transcription factor Nrf1 mediates the proteasome recovery pathway after proteasome inhibition in mammalian cells. Mol. Cell *38*, 17-28.

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